

# Probing Ion Channels and Recognition Sites of Neuronal Nicotinic Cholinergic Receptors with Novel Nicotine Affinity and Other Ligands<sup>a</sup>

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One of the fundamental problems of receptor biology concerns the interrelationships of the receptor agonist recognition site to voltage-dependent ion channels associated with excitability and other bioelectric phenomena. There are two known classes of receptors within the nervous system: those in which the ion channels are an integral component of the receptor complex comprising multiple subunits and those in which receptors are linked to ion channels via G-proteins. The former includes the nicotinic cholinergic, gabaminergic, glycinergic, and glutaminergic receptors, and comprises a superfamily with remarkable sequence homology in the  $\beta$  subunits and the seven membrane-spanning regions.<sup>1</sup> The G-coupled receptors, which are a far more extensive group for endogenous mediators, include muscarinic cholinergic, biogenic amines (catecholamines and serotonin), peptides, hormones, and growth factors. In the *Torpedo* membrane the ionic pore results from the pentameric array of the nAChR subunits.<sup>2</sup> Although neuronal nAChR receptors are composed of only  $\alpha$  and  $\beta$  subunits, lacking the  $\delta$  and  $\gamma$  subunits of the *Torpedo* nAChR, the high degree of sequence homology in uncharged segments of the membrane-spanning regions of all four subunits<sup>3</sup> is suggestive of ion-channel functional homology in all nAChRs. The issue of whether the nAChR ion channel comprises a single or multiple subunits is still unresolved.

Subtypes of nAChR, comprising  $\alpha_7$  or  $\alpha_8$  subunits and exhibiting a high affinity for  $\alpha$ -bungarotoxin and a low affinity for nicotine, have been shown to contain ligand-gated  $\text{Ca}^{2+}$  ion channels.<sup>4</sup> It remains to be determined whether the nAChR receptors in chick ciliary ganglion, which contain voltage-gated  $\text{Ca}^{2+}$  channels that may be associated with signaling function as well as synaptic transmission, comprise  $\alpha_7$  or  $\alpha_8$  subunits.<sup>5</sup>

## REGULATION OF ION CHANNELS BY G-PROTEINS

Ion conductance within nicotinic cholinergic (nAChR) and excitatory amino acid receptors results directly from conformational changes in the receptor protein

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complex, whereas  $K^+$  conductance initiated by muscarinic cholinergic receptors, such as the M2 subtype in heart muscle, is mediated via a pertussin toxin-sensitive G-protein coupled to the ion channel.<sup>6</sup> Although some controversy remains regarding a direct G-protein gating of ion channels, the most definitive evidence for the hypothesis derives from inside-outside membrane patch studies in muscarinic-sensitive heart muscle demonstrating that the application of either the GTP $\gamma$ S subunit or a purified pertussis toxin  $G_i$ -protein purified from erythrocytes results in activation of  $K^+$  channels.<sup>7,8</sup> In contrast to atrium, where a  $G_i$ -protein is involved, the muscarinic sensitive  $K^+$  channel in hippocampal neurons is coupled to  $G_o$ .<sup>9</sup>

When expressed in *Xenopus* oocytes, the  $\alpha_7$  subunit, unlike other nAChR subtypes, exhibits significant agonist-mediated  $Ca^{2+}$  conductance (TABLE 1). A major portion of the current through the  $\alpha_7$  channels is carried by  $Ca^{2+}$ , and the incoming  $Ca^{2+}$  in turn activates  $Ca^{2+}$ -dependent  $Cl^-$  conductance. Agonist activation results in a biphasic current consisting of an initial inward current through  $\alpha_7$  channels followed by an outward current through  $Ca^{2+}$ -dependent  $Cl^-$  channels.<sup>10</sup> It has been speculated that the  $\alpha_7$  homoligomer, which is present in a number of limbic

TABLE 1. Ion Permeability Associated with Various nAChR Subtypes

Subtype	Ions	Source
$\alpha_4$	$Ca^{2+}$	Ganglia
$\alpha_7$	Voltage-gated $Ca^{2+}$ $Ca^{2+}$ -dependent $Cl^-$	Hippocampus, cochlea
$\alpha_8$	Same as $\alpha_7$	Chick retina
$\beta_2$	Inhibitory	Rat dorsolateral septal nucleus
$\alpha_2$ - $\alpha_4$	Excitatory	Rat medial vestibular nucleus
	Cation selective	Cardiac ganglion
$\alpha_2$	$Cs^+$ , $Na^+$ , $Ca^{2+}$ $P_{Cl}/P_{Na} = 0.05$	

system areas, may be involved in the activation of  $Ca^{2+}$ -dependent mechanisms; however, it is difficult to reconcile this notion with the observation that  $\alpha$ -bungarotoxin was pharmacologically inactive when administered intraventricularly in high concentrations to rats.<sup>11</sup> Both an  $\alpha$ -bungarotoxin sensitive and  $\alpha$ -bungarotoxin-insensitive ( $\alpha_3\beta_4$ ) nAChR have been found in chick ciliary ganglion; the former is synaptic in origin and exhibits agonist-mediated voltage sensitive  $Ca^{2+}$  permeability, whereas the latter is monosynaptic and exhibits voltage-insensitive  $Ca^{2+}$  permeability.<sup>12</sup> Acetylcholine or nicotine-evoked currents in cultured neurons dissociated from rat parasympathetic cardiac ganglia exhibited a strong inward rectification and cation selectivity; the permeabilities of  $Cs^+$ ,  $Ca^{2+}$ , and  $Na^+$  were comparable, and the  $Cl^-$  permeability was one-twentieth of that of  $Na^+$ .<sup>13</sup> Because the response was inhibited by both mecamylamine and hexamethonium in a dose-dependent manner, it was concluded that the ACh-activated ion channels of the postganglionic neurons are mediated by nAChRs. A summary of the voltage-gated ion channels of the nAChR is presented in TABLE 1.

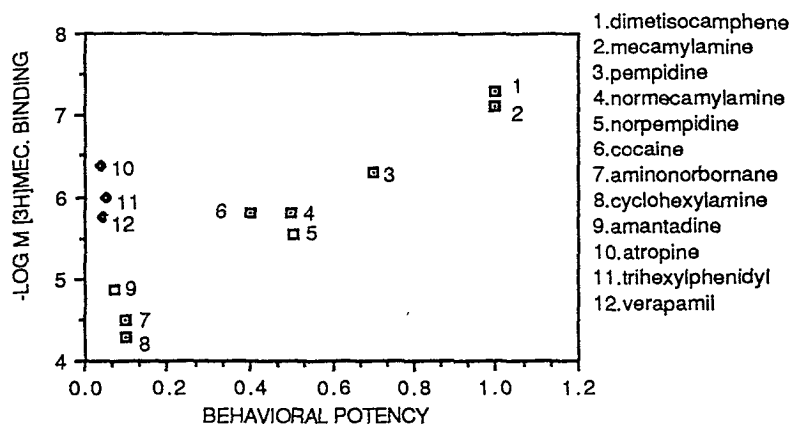


FIGURE 2A. Plot of  $\text{IC}_{50}$  for  $[^3\text{H}]$ mecamylamine binding versus antagonism of nicotine behavioral effects of various antagonists. Behavioral potency is expressed relative to mecamylamine as 1. Mice were given 10  $\mu\text{moles/kg}$  nicotine intraperitoneally followed by 50  $\mu\text{moles/kg}$  of test agent after occurrence of behavioral effects, which included prostration, tremors, seizures, straub tail, respiratory depression, and decreased motor activity. Five mice were used for each agent. Procedures for  $[^3\text{H}]$ mecamylamine binding and behavioral measurements are described elsewhere.<sup>27</sup>

binding and the ability of the agents to block nicotine-induced prostration and seizures in rats; however, a number of structurally and pharmacologically unrelated agents, including various nicotine analogues, had  $K_i$  values in the  $\mu\text{M}$  range (FIGS. 2A and 2B). Cocaine, which appears to block nicotine-induced prostration, but not seizures, has a  $K_i$  value over a magnitude greater than that for mecamylamine, whereas the muscarinic antagonists, atropine and trihexylphenidyl, are ineffective against nicotine's action.

#### COMPARISON OF $[^3\text{H}]$ MECAMYLAMINE WITH $[^3\text{H}]$ METHYLCARBAMYLCHOLINE AND $[^3\text{H}]$ (R,S)-3-PYRIDYL-1- $[^3\text{H}]$ METHYL-2-AZETIDINE BINDING IN VARIOUS REGIONS OF CALF BRAIN

Because the density of nAChRs showed marked variations in the various brain regions, a comparison of the extent of  $[^3\text{H}]$ mecamylamine with  $[^3\text{H}]$ methylcarbamylcholine ( $[^3\text{H}]$ MCC) binding may help resolve the issue concerning the significance of the  $[^3\text{H}]$ mecamylamine binding in relation to nAChR function. The aim of the study was to compare  $[^3\text{H}]$ mecamylamine binding to membranes from various calf brain regions with the binding of two different receptor ligands, one chemically related to acetylcholine and the other to nicotine. A comparison of the  $[^3\text{H}]$ MCC and  $[^3\text{H}]$ (R,S)-3-pyridyl-1- $[^3\text{H}]$ methyl-2-azetidine ( $[^3\text{H}]$ MPA) (a nicotine analogue<sup>20</sup>) binding in membranes from various calf brain regions revealed that the density of nAChR receptors was threefold greater in striatum and substantia nigra compared to frontal cortex, hippocampus, and locus coeruleus, whereas the density in the cerebellum was one-tenth that of the frontal cortex (TABLE 2). With  $[^3\text{H}]$ MPA as the ligand, total

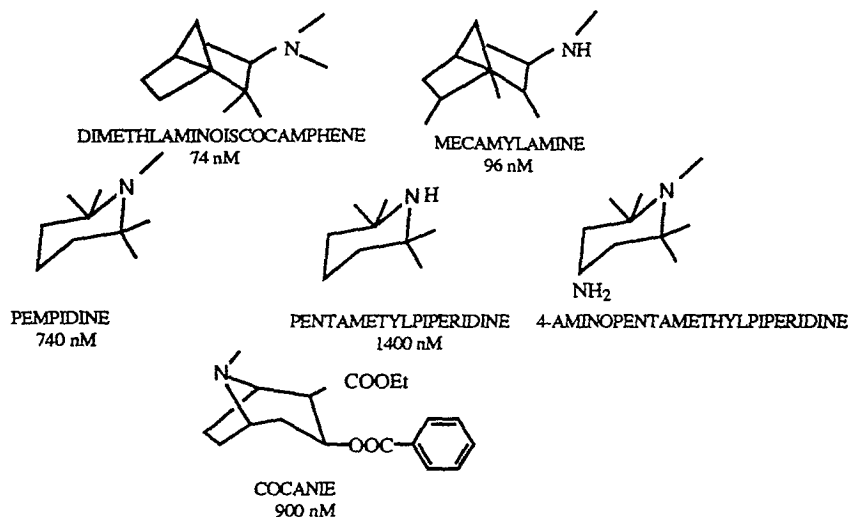


FIGURE 2B. Ion channel blockers for nicotinic receptors. Chemical structures of mecamylamine analogues and related agents.

binding in the various brain regions was comparable to that observed with [<sup>3</sup>H]MCC, with some notable exceptions. The extent of [<sup>3</sup>H]MPA binding in the cerebellum was 33% that of the frontal cortex as compared to 10% seen with [<sup>3</sup>H]MCC, whereas the density of [<sup>3</sup>H]MPA binding in the locus coeruleus was 40% that in the frontal cortex. [<sup>3</sup>H]MCC binding in the substantia nigra was greater than that seen in striatum, whereas [<sup>3</sup>H]MPA binding was slightly less in the substantia nigra than in the striatum. The difference in [<sup>3</sup>H]MCC and [<sup>3</sup>H]MPA binding in the various brain regions may be attributable to differences in the nAChR subtypes.

The saturation plots of [<sup>3</sup>H]mecamylamine binding for the various brain regions show the greatest binding in the substantia nigra, intermediate binding in the

TABLE 2. Comparison of [<sup>3</sup>H]MCC and [<sup>3</sup>H]MPA Binding in Membranes of Various Calf Brain Regions<sup>a</sup>

	[ <sup>3</sup> H]MCC		[ <sup>3</sup> H]MPA	
	fmole/mg	% Frontal Cortex	fmole/mg	% Frontal Cortex
Frontal cortex	13	—	15	—
Hippocampus	14	108	14	93
Striatum	35	270	36	242
Substantia nigra	43	330	33	219
Cerebellum	1.3	10	5	33
Locus coeruleus	11.5	90	6	40

Abbreviations: [<sup>3</sup>H]MCC: [<sup>3</sup>H]methylcarbamylocholine; [<sup>3</sup>H]MPA: (R,S)-3-pyridyl-1-[<sup>3</sup>H]methyl-2-azetidine, an analogue of nicotine.<sup>20</sup>

<sup>a</sup>The results are an average of three separate experiments run in triplicate and agreeing within 8%.

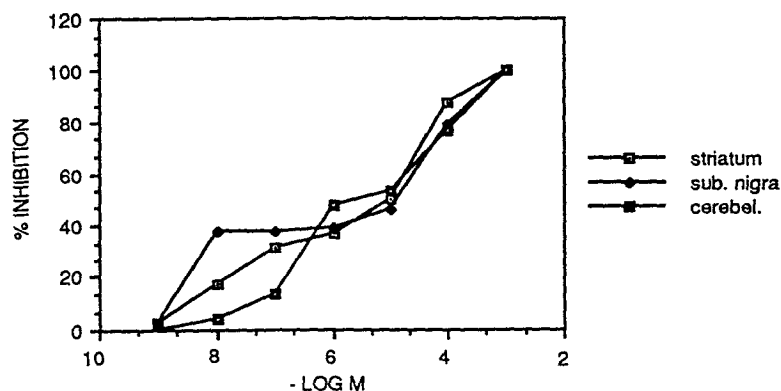


FIGURE 3A. Saturation analysis of  $[^3\text{H}]$ mecamylamine binding in various calf brain regions.

striatum, and little or no binding in the cerebellum within the 1–10 nM range (FIGS. 3A and 3B). At concentrations of 100 nM and greater no difference was found among the various brain regions.  $[^3\text{H}]$ MCC binding differed markedly among various brain regions, with the highest density in the substantia nigra and striatum and the lowest in the cerebellum (TABLE 2). The rank order of the densities of  $[^3\text{H}]$ MCC binding for the various tissues is similar to the rank order of densities for  $[^3\text{H}]$ mecamylamine binding in the lower but not upper concentration range. Because a variety of alkaloids, including nicotine analogues, have  $K_i$  values in the micromolar range (FIG. 2A) but do not block the behavioral effects of nicotine, one might infer that only  $[^3\text{H}]$ mecamylamine binding occurring near the submicromolar range is reflective of nAChRs. It also appears that the  $[^3\text{H}]$ mecamylamine binding curve is biphasic, suggestive of lower and higher affinity sites. It remains to be seen whether the lower affinity binding is of any functional significance.

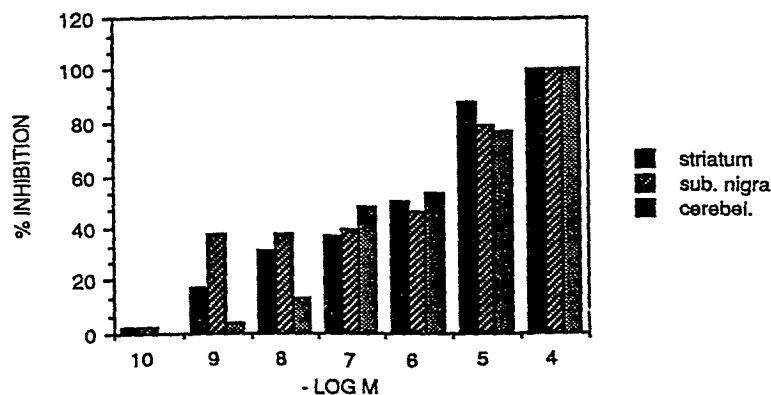


FIGURE 3B.  $[^3\text{H}]$ Mecamylamine binding in various calf brain regions. Bar graph representation of FIGURE 3A.

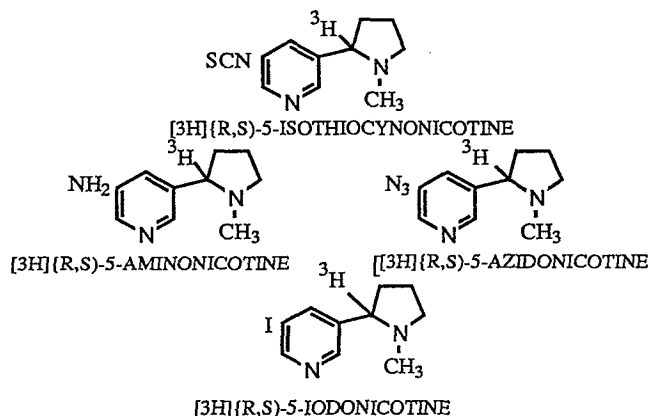


FIGURE 4. Chemical structures of [ $^3\text{H}$ ]-labeled nicotine affinity and photoaffinity ligands.

#### CHEMICAL NATURE OF THE nAChR AGONIST RECOGNITION SITE

The techniques of affinity labeling and construction of chimeric receptor subunits have been used to map the ligand recognition site of muscle and neuronal nAChRs. The nAChR is known to contain a readily reducible sulfhydryl site adjacent to the negative site for acetylcholine binding;<sup>21</sup> alkylating agents, such as [ $^3\text{H}$ ]-4-(N-maleimido)benzyltrimethylammonium, have been used to identify and purify the  $\alpha$ -subunit of the *Torpedo* nAChR.<sup>22</sup> The site of interaction of the sulfhydryl agent has been shown to be Cys192 and Cys193, which normally form a disulfide bond; this region in the vicinity of the disulfide bond is conserved in both muscle and neuronal nAChRs.<sup>23</sup> In addition to the Cys192 and Cys193 of the  $\alpha$ -subunit, a number of other amino acids appear to play a role in agonist binding and function, including Tyr89,<sup>24</sup> Trp149,<sup>25</sup> and Tyr198.<sup>2</sup> Recently, site-directed mutagenesis of the *Torpedo*  $\delta$  subunit has demonstrated the involvement of the acidic residues, Asp180 and Glu189, for acetylcholine binding.<sup>25</sup> A schematic model for the nAChR has been proposed in which acetylcholine is bound in the Asp180–Glu189 region and surrounded by an array of other amino acids (Cys192, Cys193, Tyr93, Trp149, and Tyr198) that contribute to its binding.<sup>26</sup> The structural perturbation resulting from the interaction of acetylcholine with this array of amino acids is then transmitted to the ion channel to somehow regulate ion conductance.

#### (R,S)-5-ISOTHIOCYANONICOTINE AND [ $^3\text{H}$ ]NICOTINOID PHOTOAFFINITY LIGANDS AS TOOLS FOR PROBING nAChRs

As part of an effort to examine the specificity of nicotine for nAChR subtypes and determine its sites of interaction a series of unlabeled and [ $^3\text{H}$ ]-labeled nicotine affinity ligands were prepared<sup>26,27</sup> (FIG. 4). The most useful ligand was (R,S)-5-isothiocyanatonicotine (SCN-nic), which was found to irreversibly inhibit the binding of [ $^3\text{H}$ ]MCC to brain membranes in the nanomolar range. SCN-nic also inhibited mouse brain nicotinic receptors *in vivo* in a dose-dependent manner, the inhibition

being 50% at a dose of 20 mmoles/kg. Behavioral studies in mice revealed that SCN-nic had less than one-fifth the potency of nicotine in producing muscle weakness and seizures with a considerably greater duration of action. Other nicotinicoids included three photoaffinity agents: [ $^3\text{H}$ -1'] (R,S)-5-azidonicotine, [ $^3\text{H}$ -1'] (R,S)-5-iodonicotine, and [ $^3\text{H}$ -1'] (R,S)-5-aminonicotine.

Photolysis of iodoaryl compounds results in cleavage of iodide to form an aryl radical,<sup>28</sup> which is capable of extracting H from SH, NH<sub>2</sub>, and OH groups of amino acids, whereas photolysis of aryl amines is believed to result in the extraction of an amino H to form a aryl imino cation.<sup>29</sup> Photolysis of membrane preparations in the presence of 10  $\mu\text{M}$  [ $^3\text{H}$ ] (R,S)-5-iodonicotine, [ $^3\text{H}$ ] (R,S)-5-azidonicotine, and [ $^3\text{H}$ ] (R,S)-5-aminonicotine resulted in 46, 4, and 23% inhibition of [ $^3\text{H}$ ]MCC binding, respectively, as compared with 13, 42, and 2% inhibition of [ $^3\text{H}$ ]3-quinuclidinylbenzilate ([ $^3\text{H}$ ]QNB).

#### EFFECT OF DITHIOTHREITOL ON INHIBITION OF [ $^3\text{H}$ ]MCC BINDING BY NICOTINE AFFINITY LIGANDS

The IC<sub>50</sub> for irreversible inhibition of [ $^3\text{H}$ ]MCC SCN-nic was recently shown to be  $1 \times 10^{-7}$  M in the absence of dithiothreitol (DTT) and  $2 \times 10^{-9}$  M in its presence.<sup>27</sup> It was also observed that alkylation of SH groups by N-ethylmaleimide did not alter the inhibition of [ $^3\text{H}$ ]MCC binding by SCN-nic (Fig. 5). Pretreatment of calf brain cortical membranes with DTT prior to photolysis also resulted in a marked increase in the inhibition of [ $^3\text{H}$ ]MCC binding by (R,S)-5-azidonicotine with no effect on the inhibition of [ $^3\text{H}$ ]QNB binding. The findings are consistent with the notion that a vicinal disulfide bond is involved in agonist binding to brain nAChRs, and although the presence of free SH groups enhances the covalent interaction of SCN-nic, they are not required for the covalent interaction of SCN-nic. The inability

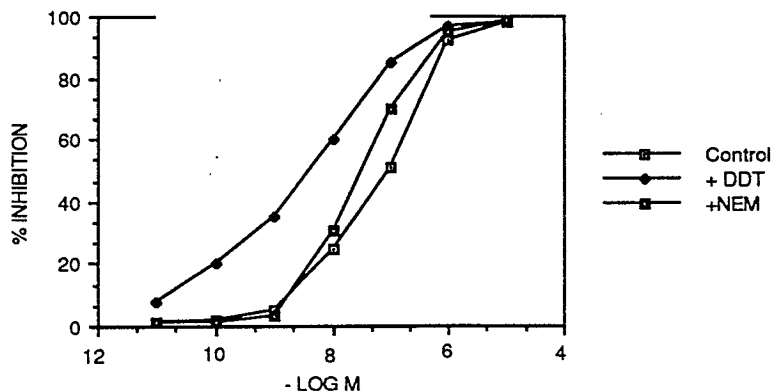


FIGURE 5. Effect of dithiothreitol (DTT) and N-ethylmaleimide (NEM) on the irreversible inhibition of [ $^3\text{H}$ ]MCC binding by SCN-nic. Membranes of calf frontal cortex were incubated with either 1 mM DTT or 3 mM NEM for 30 min at room temperature, centrifuged and washed once with 0.04 mM sodium phosphate, pH 7.0, exposed to varying concentrations of SCN-nic, and washed three times before determining [ $^3\text{H}$ ]MCC binding. The results are an average of three separate experiments in triplicate, agreeing within 7%. Experimental details are described elsewhere.<sup>18</sup>

of DTT to alter the photolytic inhibition of (R,S)-5-iodononnicotine and (R,S)-5-aminononnicotine also supports the notion that SH groups are not involved in the reactivity of the photoreactive nicotinoids with nAChRs. Photolabeling with [ $^3$ H]nicotine has been shown to primarily involve Tyr-198.<sup>30</sup> Both the pyridine<sup>31</sup> and pyrrolidine<sup>32</sup> rings of nicotine appear to participate in radical mediated photoreactions; however, information on the nature of the reactions involved is lacking. The present finding that the presence of either an iodo or amino group in position 5 significantly increases the photoreactivity of the nicotine molecule supports the notion that the pyridine ring is involved.

#### EFFECT OF DITHIOTHREITOL TREATMENT ON IRREVERSIBLE INHIBITION OF [ $^3$ H]MCC AND [ $^3$ H]MPA BINDING BY SCN-NIC

To further explore the involvement of SH groups in the ligand binding region of nAChRs, a study was undertaken to determine the effect of DTT treatment on

TABLE 3. Effect of Dithiotreitol on SCN-nic Inhibition of [ $^3$ H]MCC and [ $^3$ H]MPA Binding to Membranes from Various Calf Brain Areas<sup>a</sup>

Brain Area	[ $^3$ H]MCC IC <sub>50</sub> nM			[ $^3$ H]MPA IC <sub>50</sub> nM		
	Control	DTT	Change	Control	DTT	Change
Frontal cortex	100	2	50-fold	10	5	2-fold
Striatum	98	10	10-fold	100	30	3.3-fold
Substantia nigra	90	12	7.5-fold	80	60	1.3-fold
Locus coeruleus	75	10	7.5-fold	80	80	0

<sup>a</sup>Membranes were exposed to 2 mM DTT and washed once with 0.04 mM sodium phosphate, pH 7.5. They were then exposed to various concentrations of SCN-nic, washed thrice, and measured for [ $^3$ H]MCC and [ $^3$ H]MPA binding. The results are an average of three separate experiments run in triplicate and agreeing within 8%. DTT, dithiotreitol; SCN-nic, (R,S)-5-isothiocyannonicotine.

irreversible inhibition of [ $^3$ H]MCC and [ $^3$ H]MPA binding by SCN-nic for various calf brain regions. The enhancement of SCN-nic inhibition by DTT was significantly greater for [ $^3$ H]MCC than for [ $^3$ H]MPA binding; the frontal cortex showed a 50-fold enhancement and other brain regions from 7.5–10-fold (TABLE 3). Only a slight enhancement in the irreversible inhibition was noted with [ $^3$ H]MPA as the ligand. The findings are consistent with the notion that the two radioligands differ in their affinity to nAChR subtypes.

#### NEURONAL nAChR SUBTYPES THAT ARE RESISTANT TO SULFHYDRYL REAGENTS

Although the disulfide bond at positions 192 and 193 of the  $\alpha$  subunit is required for activation of the skeletal muscle nAChR, the requirement for the vicinal cystine residue appears to be different for the various neuronal nAChR subtypes. It has been shown that agonist activation of an inhibitory nAChR in the rat dorsolateral septal nucleus is unaffected by DTT followed by alkylation of disulfide bonds by bromoace-



TABLE 4. Effect of Dithiotreitol on [<sup>3</sup>H]MCC and [<sup>3</sup>H]MPA Binding to Membranes from Various Calf Brain Regions<sup>a</sup>

Brain Area	[ <sup>3</sup> H]MCC (pmole/mg)			[ <sup>3</sup> H]MPA (pmole/mg)		
	Control	DTT	% Change	Control	DTT	% Change
Frontal cortex	12.2	6.2	-49	11.4	5.2	-54
Striatum	32.5	15.0	-54	32.0	33.0	+3
Substantia nigra	42.0	26.5	-40	40.2	36.5	-10
Locus coeruleus	11.2	11.3	0	6	6.2	0

<sup>a</sup>Membranes were exposed to 2 mM dithiotreitol (DTT) for 30 min and washed twice with 0.04 mM sodium phosphate, pH 7.5, prior to determining receptor binding. The results are an average of three separate experiments run in triplicate and agreeing within 7%.

tylcholine; however, an excitatory nAChR in the rat medial vestibular nucleus is inhibited upon treatment with DTT.<sup>33</sup> Because the septal nucleus does not appear to contain  $\alpha_2$ ,  $\alpha_3$ , or  $\alpha_4$  subunits, but does contain  $\beta_2$  subunits,<sup>34</sup> it was suggested that a novel inhibitory receptor contains a  $\beta_2$  subunit.

To explore further the possibility that [<sup>3</sup>H]MCC and [<sup>3</sup>H]MPA differ in their affinities for nAChR subtypes we examined the effect of DTT on [<sup>3</sup>H]MCC and [<sup>3</sup>H]MPA binding in various regions of calf brain (TABLE 4). With [<sup>3</sup>H]MCC as the ligand, binding was inhibited 40–50% in all brain regions except the locus coeruleus. With [<sup>3</sup>H]MPA as the ligand, the only brain region inhibited was the frontal cortex. The findings are suggestive of differences in the nAChR subtypes in the various brain regions and in the affinity of the two ligands for the subtypes.

#### EFFECT OF SCN-NIC ON [<sup>3</sup>H]MECAMYLAMINE BINDING

A study was undertaken to determine if SCN-nic affected [<sup>3</sup>H]mecamylamine binding to calf brain membranes. Calf brain membranes were treated with  $1 \times 10^{-5}$  M SCN-nic to completely inhibit [<sup>3</sup>H]MCC binding, and then assayed for [<sup>3</sup>H]mecamylamine binding. A plot of the % inhibition of binding versus concentration of unlabeled mecamylamine showed only 25% inhibition at  $1 \times 10^{-5}$  M and none at  $1 \times 10^{-6}$  M (data not shown). It was also found that [<sup>3</sup>H]mecamylamine binding was unchanged in membranes photolytically exposed to 5-azidonicotine and the other nicotine photoaffinity ligands. This finding indicates that the site of interaction of

TABLE 5. Reaction of [<sup>3</sup>H]SCN-nic Labeled Proteins from Rat Brain Cortex with Antibodies of nAChR Subunits<sup>a</sup>

nAChR Subunit	Reaction
$\alpha_3$	+
$\alpha_4$	+
$\alpha_7$	+
$\alpha_8$	-
$\beta_2$	-

<sup>a</sup>Membranes from whole rat brain were labeled with [<sup>3</sup>H]SCN-nic, separated by SDS-gel electrophoresis, transferred to nitrocellulose membranes, and exposed to 1:5000 dilution of antibodies. Immunoblots were compared with [<sup>3</sup>H]SCN-nic labeled bands from radiograms of duplicate gels. The chicken monoclonal antibodies were a gift of Jon Lindstrom.

mecamylamine with neuronal nAChRs is distinct from that of the nicotinic recognition site. Furthermore, occupancy of the recognition site by a variety of covalent nicotine ligands does not affect the interaction of mecamylamine with the nAChR receptor. Although there are limitations to the use of [ $^3$ H]mecamylamine to determine the allosteric sites of nAChRs, mecamylamine and related nicotine antagonists with  $K_i$  values of 10  $\mu$ M or lower appear to correlate reasonably well with their ability to antagonize the pharmacological effects of nicotine.

#### REACTION OF ANTIBODIES OF nAChR SUBUNITS TO [ $^3$ H]SCN-NIC LABELED PROTEINS

A study was performed to determine which nAChR subunits were labeled by [ $^3$ H]SCN-nic. Membranes from whole rat brain were labeled with [ $^3$ H]SCN-nic, separated by acrylamide SDS-gel electrophoresis, transferred to nitrocellulose membranes, and exposed to 1:5000 dilution of the antibodies. Immunoblots obtained with various monoclonal antibodies derived from chicken nAChR subunits (gift of Jon Lindstrom) were compared with specifically [ $^3$ H]SCN-nic labeled bands on radiograms of duplicate gels. A positive reaction was obtained with  $\alpha_3$ ,  $\alpha_4$  and  $\alpha_7$  subunits, whereas  $\alpha_8$ , and  $\beta_2$  subunits were negative (TABLE 5). The findings support the notion that [ $^3$ H]SCN-nic has selectivity for some of the neuronal nAChR subunits.

In summary, some novel affinity and photoaffinity nicotine analogues have been utilized to examine the receptor binding characteristics of membranes prepared from various calf brain regions. A re-examination of the use of [ $^3$ H]mecamylamine as a probe for the ion channel of nAChRs suggests that its usefulness is limited to analogues of mecamylamine and pempidine with  $K_i$  values in the micromolar range. Immunoblot studies with [ $^3$ H]SCN-nic and the differences observed in the reactivity of the nicotine affinity ligands and the binding affinities of [ $^3$ H]MCC and [ $^3$ H]MPA in various calf brain regions suggest that various ligands may prove useful in characterizing nAChR subtypes.

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